Six amino acid substitutions in the carboxyl-transferase domain of the plastidic acetyl-CoA carboxylase gene are linked with resistance to herbicides in a *Lolium rigidum* population

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Summary

- The molecular basis of an acetyl-CoA carboxylase (ACCase) target-based resistant *Lolium rigidum* population (WLR 96) was studied here.
- The carboxyl-transferase domain of the plastidic ACCase gene from resistant individuals was amplified by PCR and sequenced. The DNA sequences were aligned and compared with a susceptible population.
- Six amino acid substitutions were identified in the resistant population. The substitution Ile-2041-Asn, known to confer resistance to ACCase-inhibiting herbicides aryloxyphenoxypropionate (APP) in *Alopecurus myosuroides*, was identified in most resistant plants but it is always linked with other amino acid substitutions. This was confirmed by a cleaved amplified polymorphism (CAP) marker and an allele-specific PCR. The sole amino acid substitution Ile-2041-Asn was not found in this population. It is likely this mutation evolved later among individuals already possessing the other substitutions. Three haplotypes were identified from the resistant population based on the six amino acid combinations, and two are linked with herbicide resistance in this population.
- The multiple amino acid substitutions including the Ile-2041-Asn form the molecular basis endowing a high degree of resistance to ACCase-inhibiting herbicides in this *L. rigidum* population.

Key words: ACCase gene, amino acid substitution, evolution, haplotype, herbicide resistance, *Lolium rigidum*, mutation.

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Introduction

Acetyl-CoA carboxylase (ACCase) (EC.6.4.1.2), by catalyzing the carboxylation of acetyl-CoA to produce malonyl-CoA, is a key enzyme in fatty acid biosynthesis. In plants, two isoforms of ACCase have been found: the plastidic ACCase in chloroplasts and cytosolic ACCase in the cytosol. The cytosolic ACCase in all the plants was found as a multidomain enzyme. The plastidic ACCase in dicot plants is a multisubunit protein complex, but in grasses it is a multifunctional enzyme encoded by a large nuclear gene, containing three distinct functional domains: biotin-carboxylase (BC), biotin-carboxyl carrier protein (BCCP) and carboxyl-transferase (CT) (Gornicki *et al.*, 1994; Konishi *et al.*, 1996; Nikolau *et al.*, 2003). Two chemical classes of herbicides, aryloxyphenoxypropionate (APP) (e.g. diclofop, haloxyfop and fluazifop) and cyclohexanedione (CHD) (e.g. sethoxydim and tralkoxydim), inhibit the ACCase in grasses (Herbert *et al.*, 1996), with only the plastidic ACCase from grass species sensitive to these herbicides (Burton *et al.*, 1991;

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Table 1 Summary of the amino acid substitutions identified in the plastidic acetyl-CoA carboxylase (ACCase) gene from resistant grass populations

Amino acid substitution[a]	Grass species	Herbicides of resistance	Reference
Ile-1781-Leu	*Setaria viridis*	CHD, APP	Délye *et al*. (2002a)
Ile-1781-Leu	*S. viridis*	CHD, APP	Zhang & Devine (2000)
Ile-1781-Leu	*Alopecurus myosuroides*	CHD, APP	Délye *et al*. (2002b, 2002c)
Ile-1781-Leu	*A. myosuroides*	CHD, APP	Brown *et al*. (2002)
Ile-1781-Leu	*Lolium rigidum*	CHD, APP	Délye *et al*. (2002b)
Ile-1781-Leu	*L. rigidum*	CHD, APP	Zagnitko *et al*. (2001)
Ile-1781-Leu	*L. rigidum*	CHD, APP	Zhang and Powles (2006)
Ile-1781-Leu	*A. fatua*	CHD, APP	Christoffers *et al*. (2002)
Ile-1781-Leu	*L. multiflorum*	CHD, APP	White *et al*. (2005)
Ile-2041-Asn	*A. myosuroides*	APP	Délye *et al*. (2003)
Ile-2041-Asn	*L. rigidum*	APP	Délye *et al*. (2003)
Ile-2041-Val	*L. rigidum*	APP	Délye *et al*. (2003)
Trp-2027-Cys	*A. myosuroides*	APP	Délye *et al*. (2005)
Gly-2096-Ala	*A. myosuroides*	APP	Délye *et al*. (2005)
Asp-2078-Gly	*A. myosuroides*	CHD, APP	Délye *et al*. (2005)
ILe-1781-Leu and Gln- 1756-Glu	*L. rigidum*	CHD, APP	Zhang & Powles (2006)

APP, aryloxyphenoxypropionate; CHD, cyclohexanedione.
[a]The amino acid positions refer to the full-length sequence of the plastidic ACCase protein in *A. myosuroides* (derived from nucleotide sequence accession AJ310767).

Konishi & Sasaki, 1994). ACCase-inhibiting herbicides (APP and CHD) bind to the CT domain of the plastidic ACCase, leading to inhibition of fatty acid biosynthesis and, ultimately, plant death (Gronwald, 1991).

The ACCase-inhibiting herbicides have been used to selectively control grass weeds worldwide since the 1980s. As a consequence of their commercial success (intensive and widespread use) resistance to the herbicides has evolved in many grass weeds (reviewed by Devine & Shimabukuro, 1994; Délye, 2005; Heap, 2005). In many cases, ACCase-inhibiting herbicide resistance is the result of reduced sensitivity of the plastidic form of the enzyme, although enhanced metabolism of those herbicides can also be selected in some cases (Devine & Shimabukuro, 1994; Devine, 1997; Devine & Shukla, 2000; Délye, 2005). For ACCase-based resistance, single amino acid substitutions in the plastidic ACCase protein have been identified as mutations endowing resistance in many grass populations (reviewed by Délye, 2005) (summarized in Table 1). Recently we found two amino acid substitutions (Ile-1781-Leu and Gln-1756-Glu) linked with resistance in two ACCase inhibitor-resistant *L. rigidum* populations, SLR 3 and SLR 31-R2 (Zhang & Powles, 2006). Ten amino acid replacements in the plastidic ACCase gene have also been found in a resistant *L. multiflorum* population (White *et al.*, 2005).

In earlier work, we characterized a *L. rigidum* population (WLR 96) with an insensitive ACCase conferring a high degree of resistance to APP herbicides (diclofop, fluazifop and haloxyfop) and a lower degree of resistance to CHD herbicides (sethoxydim and tralkoxydim), with no resistance to other groups of herbicides (Tardif *et al.*, 1996). This WLR 96 population, with a different history of herbicide usage from the two other resistant *L. rigidum* populations, SLR 3 (Tardif *et al.*, 1993) and SLR 31(Tardif & Powles, 1994), was selected by the sole use of diclofop-methyl for 10 yr (Heap & Knight, 1990). It was of interest to investigate the molecular basis for ACCase inhibitor resistance in this population. Here, we demonstrate that nucleotide changes in the CT domain of the plastidic ACCase gene resulting in multiple amino acid substitutions in the protein of the ACCase are linked with resistance to herbicides in this *L. rigidum* population.

Materials and Methods

Plant materials

The resistant population *Lolium rigidum* Gaud. (WLR 96) was selected with diclofop-methyl (APP) and originated from Meckering, Western Australia (Heap & Knight, 1990). This population has been propagated for many generations in our research program. A well characterized susceptible population, VLR 1, was used as control. The VLR 1 population is well known to be herbicide-susceptible and has never been exposed to any herbicides. Seedlings of the two populations were cultivated in a growth chamber with 12 h light : dark, 20 : 15°C and 75% RH. When at two-leaf stage, seedlings were sprayed with commercial formulation of the herbicide diclofop-methyl at the recommended rate (375 g ha^{-1}). All the susceptible VLR 1 seedlings (as control) were killed by this treatment, whereas the great majority of the WLR 96 seedlings survived the treatment and no visible damage could be seen. Twenty days after treatment, leaves from individual survivors were collected, wrapped separately and stored at −80°C for DNA extraction. The susceptible seedlings for DNA extraction were harvested as bulked material (at least 50 seedlings bulked) without herbicide treatment. Therefore, molecular information from this bulked susceptible population VLR 1 plant material served as control for the purpose of present study.

DNA extraction and PCR amplification of CT domain of the plastidic ACCase gene

The micro-CTAB method was used to extract DNA from single plants for PCR as described (Zhang & Powles, 2006). The CTAB method was used to extract DNA from bulked plant material.

Preliminarily, PCR amplified DNA fragments from genomic DNA of bulked seedlings of WLR 96 and VLR 1 with primers designed on the sequence of the *Setaria viridis* plastidic ACCase gene (X. Zhang, unpublished) including the entire CT domain section. To eliminate possible introns within this sequence section, RT-PCR from total RNA of the resistant and susceptible plants was also used in the preliminary experiments. The 1900 bp PCR fragments were cloned into T-vector (Invitrogen). The plasmids containing the PCR fragment insert were sequenced. The sequences from amplified genomic DNA or cDNA by RT-PCR were compared with the plastidic ACCase gene from *S. viridis* (accession number AF294805), *Alopecurus myosuroides* (accession number AJ310767) and partial sequences from *L. rigidum* (accession number AF359513–16). This confirmed that they were CT domain sequences from the plastidic ACCase gene. New primers were then designed to study allelic variations of individual plants based on the sequences of *L. rigidum*. Two primers, acclr9 (5′-ATGGTAGCCTGGATCTTGGACATG) and acclr6 (5′-GGAAGTGTCATGCAATTCAGCAA), were used to amplify a 1600 bp fragment for sequencing from resistant and susceptible plants, covering nearly the entire CT domain without any intron (equivalent to nucleotide sequence 5086–6687 of the *A. myosuroides* ACCase gene accession AJ310767).

Sequencing of PCR-produced DNA fragments of the plastidic ACCase CT domain

Initially, three plants of the resistant population were used for sequencing experiments. The PCR fragments were directly sequenced from both ends using the ABI Big Dye Terminator system with the same primers as for the PCR and two other primers in the middle of the fragment after purification with Qiagen Gel Extraction kit (Qiagen GmbH, Hilden, Germany). Sequence analysis and alignments were performed using online service programs provided by the Australian National Genomic Information Service (ANGIS; www.angis.org.au). All the sequences were visually rechecked with chromatogram files. Any uncertain sequences, or heterozygous individuals, were re-sequenced from both strands. The heterozygotes were recognized by double nucleotide peaks present at the same position on the sequence chromatogram by forward and reverse sequencing. To distinguish heterozygous haplotypes, the PCR-produced DNA fragments were cloned into T-vector using an Invitrogen T-vector cloning kit. At least two clones whose plasmids had been examined by restriction endonuclease containing the right inserts were sequenced for each haplotype.

DNA fragments of plastidic ACCase CT domain from additional resistant individual plants were sequenced when initial sequences showed a distinct result compared with the sequences of the susceptible population. A total of 14 individual resistant plants were sequenced. The DNA sequences were deposited in the GenBank Nucleotide Sequence Database (accession numbers DQ184633 to DQ184647). The nucleotide positions in the DNA sequence from *L. rigidum* used in this paper thus refer to these DNA sequences. However, for convenience of comparison, the derived amino acid positions of these DNA fragments refer to the corresponding position in the full-length plastidic ACCase protein derived from *A. myosuroides* nucleotide sequence accession AJ310767.

Screening of susceptible individuals from within the WLR 96 population

Comparison of the CT domain sequences between resistant and susceptible individuals revealed a number of amino acid differences. It was therefore important to rule out the possibility that these amino acid differences simply reflect their geographic diversity (the population WLR 96 originates 3000 km away from the susceptible population VLR 1). This was achieved by isolating the small number of susceptible individuals existing within the WLR 96 population (resulting from segregation between heterozygous individuals). Some 200 plants from WLR 96 were individually labeled and one leaf taken from each individual for DNA extraction, and then the plants were sprayed with fluazifop-butyl (APP) at the recommended rate of 106 g ha^{-1}. The leaf sample from each plant was genotyped (see the following section for details) with the cleaved amplified polymorphism (CAP) marker. Of a total of 200 treated seedlings of the WLR 96 population, 197 were found to be resistant, with only three seedlings clearly identified as susceptible. Combined leaf material from these three susceptible seedlings was used for DNA extraction and the CT domain of the plastidic ACCase was amplified and sequenced. This sequence was designated as WLR 96-S to distinguish it from the classic susceptible population VLR 1.

Cleaved amplified polymorphism (CAP) marker for genotyping

The mutation at nucleotide position 1160 causing an Ile-2041-Asn substitution results in a restriction endonuclease *Eco*R I site change. As a result, the *Eco*RI site is removed in the resistant allele. The primers acclr4 (5′-ATATATTGAGGT-GGCTCAGCTA) and acclr6 (5′-GGAAGTGTCATGCAA-TTCAGCAA) were used to amplify a 900 bp DNA fragment, and then *Eco*RI enzyme and buffer were added to the PCR tube and incubated at 37°C for 3 h. CAP was scored from agarose gel separation. Following *Eco*RI digestion, the homozygous resistant plants showed only one uncut 900 bp DNA band on the final agarose gel image. By contrast, homozygous-susceptible plants (VLR1) with the Ile-2041 alleles showed two DNA bands of 410 and 490 bp, respectively. For heterozygous individuals, all the three DNA bands are shown. This *Eco*RI digestion therefore provides a clear molecular marker for plants with the Asn-2041 substitution. In total, 64 resistant plants of WLR 96 were analyzed with this marker. In another experiment, 198 seedlings from WLR 96 were tested with this method.

Allele-specific PCR

In order to investigate the heterozygote's haplotypes and their distributions in the WLR 96 population, an allele-specific primer ASPS10r (5′T**T**AGAAACACCT**T**CAAGGTCATCT**C**) (reverse complementary of nucleotide position 647–671) was designed specifically to match haplotype A from the susceptible population at three nucleotide positions (in bold letters) (although the last base C at the 3′-end is the most important match for the specific binding). Together with primer acclr9, the primer ASPS10r could amplify a 703 bp DNA fragment from the susceptible plants and the heterozygous plants containing the haplotype A, but not from the plants containing only haplotype B or C. The PCR was performed in 25 µl reaction mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.2 µM of each primer, 50 ng of DNA template and one unit of Taq DNA polymerase (from Bioline, London, UK). The cycling program consisted of 35 cycles of 30 s at 94°C, 30 s at 57°C, 30 s at 72°C followed by a 4 min denaturation at 94°C. The PCR product was checked on a 1.5% agarose gel in TAE buffer. The PCR was repeated at least three times in separate experiments. Although this allele-specific PCR was most specific to the nucleotide 647-C (reverse code CGA for Arg), it could be used to represent haplotype A since all those five nucleotides in haplotype A were found linked in all the sequences of the CT domain in this study.

Results

Sequencing results and detection of polymorphisms in the CT domain of plastidic ACCase gene from the resistant population WLR 96 and the susceptible population VLR 1

DNA fragments (1600 bp) of the CT domain of the plastidic ACCase gene from single plants were amplified by PCR and directly sequenced from both ends. Thus, the DNA described here refers to the CT domain sequences of the plastid ACCase of *L. rigidum* with GenBank accession numbers DQ184633 to DQ184647. The reference sequence from the susceptible *L. rigidum* (VLR 1) was earlier deposited in GenBank (accession number AY995232).

In total, 14 individual resistant plants were used for PCR amplification and sequencing, with bulked susceptible plants as control. A single DNA fragment was amplified from individuals with a predicted size of 1600 bp. The CT domain sequences of the plastidic ACCase gene from resistant plants were aligned and compared with susceptibles (the sequence alignment in Table S1). This revealed a total of 33 single nucleotide polymorphisms (SNPs) among them, including 20 synonymous and 13 nonsynonymous nucleotide substitutions (see Table S1). The nonsynonymous SNP positions and resulting amino acid substitutions are shown in Table 2. Among the 13 nonsynonymous SNPs, three found only in susceptible plants (at 802, 851 and 874) and four found in susceptible plants and the heterozygous plants 9 and 12 (at 875, 1013, 1155 and 1199) are considered as random variations and unrelated to resistance, although these nucleotides in resistant plants are consistent and associated with other SNPs. The remaining six nonsynonymous SNPs, at nucleotide positions 139, 647, 659, 670, 1072 and 1160, were found only in resistant plants (compared with the susceptible plants). These six SNPs are therefore linked with resistance in the plants. The SNP at 1160 was found in 12 of the 14 resistant plants (except for plants 32 and 41). The other five SNPs were present in all the resistant plants and were always linked together (copresent). Table 2 shows that the nucleotide transversion at 1160 was independent of the other linked nucleotide replacements as it displayed heterozygosity in comparison with the homozygosity of other substitutions (in plants 1, 3, 14 and 18). The derived amino acid substitution caused by the T-1160-A change corresponds to the Ile-2041-Asn in the full-length sequence of the plastidic ACCase protein from *A. myosuroides*. This single amino acid substitution of Ile-2041-Asn is linked with resistance to APP herbicides in an *A. myosuroides* population (Délye *et al.*, 2003). However, none of the DNA sequence analyzed in the WLR 96 population displays the sole single nucleotide substitution at position 1160.

Comparison of the CT domain sequence between two susceptible populations (WLR 96-S and VLR 1)

In our laboratory, population VLR 1 is well characterized and always used as a susceptible control. However, VLR 1 originates 3000 km away from the WLR 96 population. To clarify if the multiple SNPs between WLR 96 resistant individuals and the susceptible VLR 1 population are truly molecular differences associated with herbicide resistance rather than the geographical variation of the two populations, susceptible individuals were isolated from within the WLR 96 population. Comparison of these two susceptible CT domain sequences (WLR 96-S and VLR 1) showed a total of 23 SNPs,

Table 2 The nonsynonymous single nucleotide polymorphisms (SNPs) and resulting amino acid substitutions in the carboxyl-transferase (CT) domain of plastidic acetyl-CoA carboxylase (ACCase) in resistant plants of *Lolium rigidum* population WLR 96 in comparison with two susceptible populations

Nucleotide position[a]	**139**	**647**	**659**	**670**	802	851	874	875	1013	**1072**	1155	**1160**	1199	GenBank accession number
SNP alleles[b]	**T, A**	**G, C**	**A, C**	**A, C**	A, G	A, G	G, C	A, G	A, T	**T, A**	A, T	**T, A**	C, T	
Amino acid position of *Alopecurus myosuroides*[c]	**1701**	**1870**	**1874**	**1878**	1922	1938	1946	1946	1992	**2012**	2039	**2041**	2054	AJ310767
WLR96-1	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn/Ile**	Ile	DQ184633
WLR96-2	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184634
WLR96-3	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn/Ile**	Ile	DQ184635
WLR96-4	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184636
WLR96-5	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184637
WLR96-6	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184638
WLR96-8	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184639
WLR96-9	**Met/Leu**	**Pro/Arg**	**Ala/Glu**	**His/Asn**	Ser	Lys	Glu/Gly	Glu/Gly	Asp/Val	**Met/Leu**	Glu/Asp	**Asn/Ile**	Ile/Thr	DQ184640
WLR96-10	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn**	Ile	DQ184641
WLR96-12	**Met/Leu**	**Pro/Arg**	**Ala/Glu**	**His/Asn**	Ser	Lys	Glu/Gly	Glu/Gly	Asp	**Met/Leu**	Glu	**Asn/Ile**	Ile/Thr	DQ184642
WLR96-14	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn/Ile**	Ile	DQ184643
WLR96-18	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Asn/Ile**	Ile	DQ184644
WLR96-32	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Ile**	Ile	DQ184645
WLR96-41	**Met**	**Pro**	**Ala**	**His**	Ser	Lys	Glu	Glu	Asp	**Met**	Glu	**Ile**	Ile	DQ184646
WLR96-S[d]	**Leu**	**Arg**	**Glu**	**Asn**	Ser/Gly	Lys/Arg	Glu/Gly	Glu/Gly	Asp/Val	**Leu**	Glu	**Ile**	Thr	DQ184647
VLR 1 (S)	**Leu**	**Arg**	**Glu**	**Asn**	Ser/Gly	Lys/Arg	Glu/Gly	Glu/Gly	Asp/Val	**Leu**	Glu/Asp	**Ile**	Ile/Thr	AY955232

Bold text, six SNPs, and amino acid linked with herbicide resistance.
[a]Nucleotide position: the numbers refer to the partial sequences of the plastidic ACCase gene in the GenBank accession number listed in the table.
[b]The nucleotides shown in the DNA sequences are from the susceptible and the resistant plants.
[c]Amino acid position derived from the full-length chloroplastid ACCase gene of *A. myosuroides* (EMBL accession number AJ310767).
[d]WLR96-S is the sequence from a few susceptible plants isolated from the WLR 96 population.

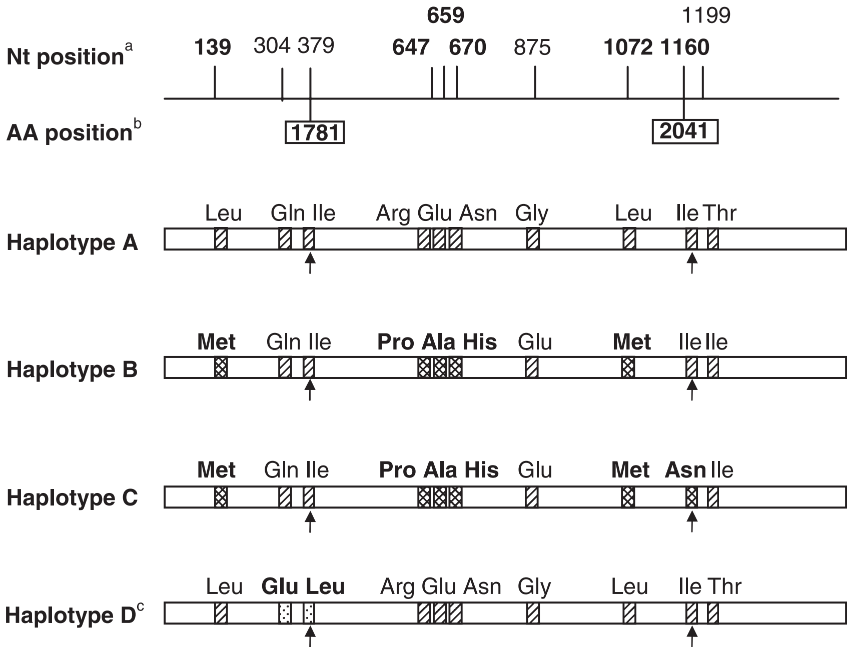

Fig. 1 Haplotypes identified from resistant population *Lolium rigidum* WLR 96, based on the combination of nonsynonymous single nucleotide polymorphisms (SNPs) and derived amino acid substitutions on the carboxyl-transferase (CT) domain of the plastidic acetyl-CoA carboxylase (ACCase) gene. The nucleotide positions 139, 647, 659, 670, 1072 and 1160 show the nonsynonymous nucleotide mutations identified in the resistant WLR 96 population. Two important amino acid positions, 1781 and 2041, are boxed. Only the derived amino acids are shown in the Fig. The different fill patterns of the amino acids highlight the differences between the haplotypes. [a]The nucleotide positions refer to the DNA sequences of the PCR fragments submitted in GenBank (accession numbers DQ184633-DQ184647). [b]The amino acid positions refer to the full-length sequence of the plastidic ACCase protein in *Alopecurus myosuroides* derived from nucleotide sequence accession AJ310767. Only two amino acid positions –1781 and –2041 were marked, as the substitutions Ile-1781-Leu and Ile-2041-Asn have been identified as a point mutation conferring resistance to ACCase-inhibiting herbicides. [c]The haplotype D was not found in the WLR 96 population. It is included here as a contrast to the other haplotypes, as it represents the major genotype in the resistant *L. rigidum* populations SLR 3 and SLR 31-R2.

including seven nonsynonymous and 16 synonymous SNPs (see Table S1). However, all the seven nonsynonymous SNPs (positions 802, 851, 874, 875, 1013, 1155 and 1199) were present in the bulked seedlings from the WLR 96-S and VLR 1 populations (Table 2). These SNPs could be considered as random variations within the populations. It is striking that pairwise comparison of these two CT domain sequences showed 100% similarity and 99% identity and therefore the susceptible plants from WLR 96-S and VLR 1, despite originating 3000 km apart, display virtually the same CT domain sequence. We therefore conclude that the differences between CT domain sequences from individuals of the resistant WLR 96 vs the susceptible VLR 1 (Table 2) are related to resistance rather than any geographic diversity. In this study, the susceptible population VLR 1 is a good control for the resistance population. Notwithstanding this, genetic variation clearly exists within and between different *L. rigidum* populations and caution is required in sequence comparison.

Comparison of the sequence-based haplotypes

Six amino acid substitutions resulting from the nucleotide changes at position 139, 647, 659, 670, 1072 and 1160 were identified in the resistant population WLR 96 (bold text, Table 2). For convenience in the analysis here, three haplotypes were designated based on combination of these six SNPs and derived amino acid substitutions in the CT domain (Fig. 1). In the susceptible populations VLR 1 and WLR 96-S the CT domain displayed the amino acid combination of Leu, Arg, Glu, Asn, Leu and Ile, and is defined as haplotype A. (It could be further divided into three subtypes based on other nucleotide changes; however, it is not related to the purpose of comparison between the susceptible and resistant populations in this study.) By contrast, the equivalent first five amino acid residues were replaced by Met, Pro, Ala, His and Met, in resistant plants 32 and 41, which was designated as haplotype B. In homozygous ACCase herbicide-resistant individuals (plants 2, 4, 5, 6, 8 and 10) the six amino acids were replaced by Met, Pro, Ala, His, Met and Asn, which is defined as haplotype C (Fig. 1). The only difference between haplotype B and C is the Ile-2041-Asn substitution. The haplotypes A, B and C represent the genotypes in population WLR 96. Haplotype D is presented in the figure for a reference, as this haplotype represents the major resistant genotype of two resistant *L. rigidum* populations (SLR 3 and SLR 31-R2) with amino acid differences at positions 1756 and 1781

(Zhang & Powles, 2006). It is emphasized that this haplotype D was not found in the population WLR 96. It is clear that the molecular basis of ACCase conferring resistance in this population is distinct, indicating that APP and CHD herbicides target different sites in the ACCase protein.

Both haplotypes A and B display an Ile-2041 and therefore could contribute to heterozygosity at this locus in the resistant population. Further sequence analysis of the cloned PCR fragments from the heterozygotes revealed that the heterozygous plants 9 and 12 were composed of two distinct haplotypes A and C. The heterozygous plants 1, 3, 14 and 18 were composed of haplotypes B and C. It is likely that the Ile/Asn-2041 is an independent mutation which could have evolved later from haplotype B.

Genotyping the population using a CAP marker for the alleles at 2041

The Ile-2041-Asn substitution resulting from the nucleotide transversion from a T to A at nucleotide position 1160 results in a restriction endonuclease *Eco*RI site (-GAATTC-) change. Therefore, restriction endonuclease *Eco*RI digestion of the PCR fragments containing the mutation site is able to distinguish the Asn- from Ile- allele. This CAP marker was used to detect this mutation in the resistant population WLR 96. Genomic DNA was individually extracted from 64 resistant plants and Table 3 shows that 64% (41 plants) of the resistant plants were homozygous (Asn/Asn), with 33% (21 plants) heterozygous (Asn/Ile). Only 3% (two plants) of plants did not have the substitution at 2041. Further sequencing was conducted with some homozygous and heterozygous resistant plants (Asn/Asn or Asn/Ile) and the small percentage of homozygous plants (Ile/Ile). The sequencing results were consistent with the CAP marker analysis (see Table S1). In contrast with the susceptible plants, the two resistant plants (32 and 41) without substitution at 2041 were found with five other linked amino acid substitutions in the CT domain (accession numbers DQ184645 and DQ184646, respectively). Since the heterozygosity at position 2041 is contributed by both haplotypes A and B, the 21 heterozygous plants could be further distinguished by detecting the presence of haplotype A or B.

In order to screen susceptible individuals existing within the WLR 96 biotype, the CAP marker was used to genotype the seedlings in an independent experiment. A similar ratio of the CAP marker scores was found in the larger population (data not shown). Therefore, it is clear that the Asn-2041 substitution exists in the majority of the WLR 96 population, as homozygous or heterozygous individuals. In resistant *A. myosuroides*, the Ile-2041-Asn substitution in the plastidic ACCase gene endows resistance to APP herbicides (Délye *et al.*, 2003). Similarly, in this resistant *L. rigidum* population, the Ile-2041-Asn substitution is likely responsible for a high degree of resistance to APP herbicides. However, it is not the only substitution in the sequences and it is always linked with other amino acid substitutions (haplotype B). Therefore, a different molecular basis is linked with resistance in this *L. rigidum* population.

Table 3 Distribution of the substitution Ile-2041-Asn and haplotypes in the resistant *Lolium rigidum* population WLR 96 (64 individuals) assayed by cleaved amplified polymorphism (CAP) marker and allele-specific PCR

Locus-2041		Haplotype A	Haplotype B	Haplotype C
Asn/Asn	41	0	0	41
Asn/Ile	21	7	14	21
Ile/Ile	2	0	2	0

Detection of haplotype A in the resistant population WLR 96

In a 25 bp region (nucleotide position 647–671), haplotype A contains three unique nucleotides different from haplotype B or C. Therefore, a PCR primer was designed for this region specifically to amplify a DNA fragment from individuals containing the haplotype A sequence. Among the 64 plants of the resistant WLR 96 population tested, only seven individuals (accounting for 11% of the WLR 96 population) produced the 703 bp DNA band with the allele-specific primers ASP10Sr/acclr9. By combining analysis with the previous CAP marker scores, the 64 individuals consist of 41 homozygous of haplotype C, two homozygous of haplotype B, seven heterozygous of haplotypes A and C, and 14 heterozygous of haplotypes B and C (Table 3). The haplotype A was exclusively produced from seven heterozygous plants and among them two plants (9 and 12) have been confirmed as heterozygotes of haplotypes A and C by cloning and sequencing the PCR fragments. No heterozygous individuals of haplotypes A and B was detected (Table 3). Base on the above ratio of different haplotypes in this population, the expected ratio of susceptible plants segregated from this population is about 1%, including homozygous haplotype A and heterozygous haplotypes A and B. This is consistent with the observation that only a very small percentage of susceptible individuals could be detected in this WLR 96 population (Tardif *et al.*, 1996). This was further confirmed in our effort to isolate susceptible individuals in the WLR 96 population by the fact that only a few plants were verified as susceptible in a large population.

Discussion

The plastidic ACCase of grass species is well established as the target enzyme inhibited by APP and CHD herbicides (Burton *et al.*, 1991). The 400-amino acid CT domain of the plastidic ACCase is known to be the ACCase herbicide binding region (Nikolskaya *et al.*, 1999). Several different amino acid substitutions in the ACCase CT domain have been identified as endowing ACCase herbicide resistance (reviewed by Délye, 2005) (summarized in Table 1). The amino acid substitution Ile-1781-Leu in the plastidic ACCase was identified in several grass species as the mutation conferring high resistance to CHD (e.g. sethoxydim) and some APP herbicides (Zhang & Devine, 2000; Brown *et al.*, 2002; Christoffers *et al.*, 2002; Délye *et al.*, 2002a; Délye *et al.*, 2002b; White *et al.*, 2005; Zhang & Powles, 2006). The Ile-2041-Asn substitution was linked with resistance to APP herbicides only in *A. myosuroides* and *L. rigidum* (Délye *et al.*, 2003). The other amino acid substitutions in the plastidic ACCase were identified mostly in *A. myosuroides*. Although in most cases single amino acid substitution is reported as endowing target site ACCase-based resistance in grass weeds in the literature, recent studies have shown that multiple amino acid substitutions can also occur (White *et al.*, 2005; Zhang & Powles, 2006). Multiple amino acid substitutions were evident in the sequences from some *L. rigidum* populations although no further analysis has been done (Zagnitko *et al.*, 2001). In a *L. multiflorum* population, White *et al.* (2005) reported 10 amino acid replacements in the plastidic ACCase gene, although they did not associate these substitutions with the resistance to CHD herbicide sethoxydim (White *et al.*, 2005). However, it is unknown whether these amino acid differences are linked with other ACCase inhibitor resistance.

We have reported that the Ile-1781-Leu and Gln-1756-Glu were identified in two ACCase inhibitor resistant *L. rigidum* populations (SLR 3 and SLR 31-R2) and linked with a high degree of resistance to CHD (sethoxydim) and some APP herbicides (Zhang & Powles, 2006). In contrast to the populations SLR 3 and SLR 31-R2, the resistant *L. rigidum* population WLR 96, selected by repeated use of APP herbicide diclofop-methyl, exhibits a high degree of resistance to APP and a lower degree of resistance to CHD herbicides (Heap & Knight, 1990; Tardif *et al.*, 1996). Here, we sequenced and analyzed the CT domain of the plastidic ACCase gene from 14 resistant plants and found that 12 plants exhibited the Ile-2041-Asn substitution. Further CAP marker analysis conducted with 64 individual plants revealed 95% of the resistant plants contained this Asn-2041 substitution. Clearly, the Asn-2041 substitution exists in the majority of resistant individuals of the WLR 96 population, as previously observed in resistant *A. myosuroides* (Délye *et al.*, 2003). Therefore, this Ile-2041-Asn substitution is likely responsible for the high degree of resistance to APP herbicides in WLR 96. However, this amino acid substitution is not the only mutation in this population. Five other linked amino acid substitutions, Leu-1701-Met, Arg-1870-Pro, Glu-1874-Ala, Asn-1878-His and Leu-2012-Met, were found to be always associated with the Ile-2041-Asn in the sequence alignment. It is unlikely that these amino acid substitutions are the result of the genetic background differences between the populations, as the comparison between the resistant and susceptible individuals from the same population showed the same amino acid substitutions. Further experiments and analysis have confirmed the linkage and presence of these five linked amino acid changes in all the resistant plants. The haplotypes C and B were identified in the resistant plants and therefore linked with ACCase-inhibiting herbicide resistance. The amino acid substitutions identified in SLR 3 and SLR 31-R2 (Zhang & Powles, 2006) were not found in this population WLR 96. Clearly, the molecular basis of ACCase conferring resistance in this population is distinct. This is probably related to their different herbicide usage history.

The linkage and coexistence of the amino acid substitutions indicates strong linkage disequilibrium during herbicide selection. It is not clear how the synchronized mutations of five amino acids in the ACCase have occurred to form the haplotype B (perhaps serving to maintain ACCase functionality). The heterozygosity status at amino acid position 2041 suggests this Ile to Asn substitution is independent and it evolved later in plants with the other linked mutations. It seems that the collaboration between haplotype B and 2041-Asn endows strong resistance in this population. Although the Asn-2041 mutation in *A. myosuroides* was the only amino acid substitution associated with ACCase herbicide resistance, this sole single mutation was not found in the resistant *L. rigidum* population WLR 96. By contrast, this substitution was always found linked with five other amino acid replacements. The haplotype B seems to be the preliminary condition for the grass to select the special mutation of Ile-2041-Asn to survive APP herbicides in this WLR 96 population. This has not been reported previously. Based on the molecular mechanism revealed in the population WLR 96, we speculate that haplotype B is a progenitor of ACCase inhibitor herbicide resistance in this population. The progenitor could confer a low-level ACCase herbicide resistance, or it could confer some (unknown) advantage in enabling survival under ACCase herbicide selection. The Ile-2041-Asn substitution could have evolved in the progenitor during ACCase herbicide selection to enhance herbicide resistance, especially to APP herbicides. However, the Ile-2041-Asn substitution alone might have some fitness cost at least in some environments and thus the individual with the sole Ile-2041-Asn substitution could not survive. It is possible that Ile-2041-Asn mutation causes a fatal protein structure change which could be overcome by the other five amino acid substitutions in the ACCase protein. This haplotype B was found in the majority of many resistant *L. rigidum* populations collected from different regions in Western Australia (X. Zhang & S. B. Powles, unpublished). This hypothesis can be further tested by comparing the herbicide susceptibility status of the 'progenitor' (haplotype B) and progenitor plus Asn-387 (haplotype C) genotypes. This experiment is under way in our group. However, it is clear that the heterozygous individuals of haplotypes A and B did not have enough resistance to survive the herbicide treatment, or it had some fitness cost. The haplotype B could be used as a 'warning' indicator of populations likely to evolve highly resistant biotypes. While in this study only a minority of individuals in the WLR 96 population were found to be homozygotes of the haplotype B, the high rate of herbicides used in the screening could have eliminated most of the plants with a low degree of resistance in previous seed propagation. Tardif *et al.* (1996) discussed the impact of the different rates of haloxyfop used in screening and hinted that some minor effect of resistant genes would have been undetected when herbicide was applied at highest rate to WLR 96 (Tardif *et al.*, 1996).

In conclusion, six amino acid substitutions in the CT domain sequences of the plastidic ACCase gene have been identified in the resistant *L. rigidum* population WLR 96, including the substitution of Ile-2041-Asn, known to endow resistance to APP herbicides in *A. myosuroides* (Délye *et al.*, 2003). However, the Ile-2041-Asn substitution alone is not found in the population. The Ile-1781-Leu substitution identified in other resistant populations was not found in this population. These six amino acid substitutions are linked with herbicide resistance in this resistant population. The haplotype B with five linked amino acid substitutions may play an important role in the evolution of ACCase herbicide resistance in *L. rigidum* populations under ACCase herbicide selection.

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Supplementary Material

The following supplementary material is available for the article online:

Table S1 ClustalW (bionav) multiple sequence alignment of plastidic acetyl-CoA carboxylase (ACCase) carboxyl-transferase (CT) domain sequences from 14 resistant individuals and two susceptible biotypes. The sequences have been deposited in GenBank and the accession number of each sequence is displayed in brackets. The nucleotide position number in the sequences is marked on the top of the sequences. The online program of ClustalW from Biomanager of ANGIS (http://bioman3.angis.org.au) was used to perform the alignment.

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